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PRINCIPAL INVESTIGATOR: William L. Stone, Ph.D.
Victor Paromov, Ph.D.
Hongsong Yang, M.D.
Min Qui, M.D.

CONTRACTING ORGANIZATION: East Tennessee State University
Johnson City, Tennessee 37614

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William L. Stone, Ph.D.; Victor Paromov, Ph.D.
Hongsong Yang, M.D. and Min Qui, M.D.

E-Mail: stone@etsu.edu

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14. ABSTRACT

The objective of this study is to develop an effective prophylactic therapy against an analog of mustard gas, 2-chloroethylethyl sulfide (CEES). The therapy for CEES-induced skin damage will be based on the topical application of antioxidant liposomes. We will use EpiDerm cultured human skin tissues as well as cultured keratinocytes as working models. HD/CEES-induced pathophysiology involves oxidative stress. Liposomes, containing both water- and lipid-soluble antioxidants, are expected to be an effective antidote/therapeutic product to protect US military and civilians from a chemical warfare agent such as HD. The therapeutic efficacy, as well as the chemical and physical stability of various antioxidant liposome formulations, will be examined to determine the optimal preparation for future testing in animal models.

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INTRODUCTION:

Sulfur Mustard (HD): The sulfur mustard (bis-2-(chloroethyl) sulfide) could effectively be used to produce casualties in the battlefield and to force opposing troops to wear full protective equipment thereby slowing down the tempo of military operations. It could also be used with devastating results against civilian targets, and such use of HD by S. Hussein's military forces in Iraq has been well documented. The extensive and slow healing skin lesions following exposure to HD would also place a heavy burden on the medical services of military and public health organizations.

Effects of HD on human skin: A characteristic of sulfur mustard exposure is the occurrence of a symptom free period of some hours post exposure. The duration of this period and the severity of the subsequent lesions are dependent upon the mode of exposure, the environmental temperature and individual to individual variations. In the first hour after exposure to mustard gas vapor or liquid no signs or symptoms are usually produced, but nausea, vomiting and eye smarting have been occasionally reported. Six to 24 hours post-exposure skin inflammation ensues followed by lesion formation and blistering.

Treatment of HD: There is no antidote or effective treatment for mustard gas intoxication.

Mechanism of CEES/HD-induced skin damage: Oxidative stress is an important mechanism for HD induced skin injury. HD and its analog 2-chloroethylethyl sulfide (CEES) induce alkylation of DNA, and rapid oxidation of intracellular proteins and lipids. CEES/HD are known also to react with the major intracellular antioxidant GSH; depleting it with a subsequent loss of protection against reactive oxygen species (ROS) and an activation of inflammatory responses. Considerable evidence suggests that HD toxicity is associated with an increased generation of damaging free radical production and promote apoptosis.

As detailed below, we have found that oxidative stress and pro-inflammatory cytokines play a key role in the toxicity of CEES. The PI's laboratory is part of a DOD funded group termed the "Advanced Medical Countermeasure Consortium". This group is systematically evaluating the overall hypothesis that oxidative stress, pro-inflammatory agents, and apoptotic cell signaling are the key factors in the toxicity of mustard gas. In recent years, there has been an enormous expansion of findings on the molecular mechanism of inflammatory responses and its relationship to oxidative stress [1-4].

We are currently taking advantage of this rich wealth of background information to help define the molecular links between inflammatory agents, oxidative stress and mustard gas toxicity. We have found that macrophages exposed to CEES have a decreased level of intracellular GSH, which is even further diminished in the presence of LPS [5, 6]. Pretreatment of the macrophages with N-acetyl cysteine (NAC) protects against the loss of intracellular GSH. NAC serves to promote the synthesis of GSH and, this is the likely mechanism for the protective effect of NAC against CEES/HD toxicity as reported by the PI's laboratory and other researchers.

The HD-induced depletion of GSH together with protein and lipid oxidation has far reaching consequences that have not been previously appreciated. HD-induced pathophysiology may occur in large part due to a disruption of redox homeostasis. Redox homeostasis is dependent upon the balance between oxidants and antioxidants. Redox sensitive gene expression is determined by the redox status of the cell. Signal transduction events induced by endogenous stimuli alter the redox state of the cell. GSH depletion influences a variety of cellular signaling process, such as activation and phosphorylation of stress kinases (JNK, p38, PI-3K) via sensitive cysteine-rich domains; activation of sphingomyelinase ceramide pathway, and activation of AP-1 and NFkB, with subsequent gene transcription [7, 8]. GSH levels are inversely related to the activity of NFkB [9]. NFkB regulates many genes involved in inflammation such as: inducible nitric oxide sythase (iNOS), proinflammatory cytokines, Il-1, TNF-alpha, interleukin 6 (IL-6), chemokine, IL-8, E-selectin, vascular cell adhesion molecule 1 (ICAM-1), and granulocyte-macrophage colony stimulating factor (GM-CSF) [8, 10].

Arroyo et al. found a dose dependent increase in TNF- alpha, IL-6, IL1 beta in SM treated human keratinocyte cells [11]. Signal transduction has also been demonstrated for CEES, wherein TNF-alpha, sphingomyelinase levels, caspase 3, 8, and 9 were all elevated [12]. Stone et al. [5] have found that inflammatory cytokines exacerbate the toxicity of CEES, as well as LPS. GSH levels are rapidly depleted by TNF-alpha. It is postulated that oxidative stress induced by CEES is further amplified by the loss of GSH and inflammatory cytokine production, thus exacerbating CEES-induced pathophysiology.

Rationale for the use of Liposomal Antioxidants: One way of reversing or preventing the ROS-induced cellular injury is via topical application of antioxidants. Vitamin E (tocopherols and tocotrienols), GSH, N-acetylcysteine (NAC), and lipoic acid are very effective antioxidants. Their antioxidative potential and importance in skin pathophysiology had been tested previously in a large number of investigations [2, 13-17]. However, the delivery of antioxidants to skin remains problematic in some cases. The intact skin allows the passage of small lipophilic substances but, in most cases, efficiently retards the diffusion of water-soluble molecules.

Liposomes are phospholipid vesicles composed of lipid bilayers enclosing an aqueous compartment. Hydrophilic molecules can be encapsulated in the aqueous spaces and lipophilic molecules can be incorporated into the lipid bilayers. Liposomes are unique in their ability to simultaneously deliver both water-soluble (in their aqueous inner space) and lipid-soluble antioxidants (in the phospholipid bilayer) to cells and tissues. They represent an ideal drug delivery system that enhances penetration of the active ingredient into the skin, localizes the drug at the site of action, and reduces percutaneous absorption.

Stone et al. [18] have reviewed the use of antioxidant liposomes in the general area of free radical biology and medicine as well as the relevant application of this technology to weapons of mass destruction.
The term “antioxidant liposome” is relatively new and refers to liposomes containing lipid soluble chemical antioxidants, water-soluble chemical antioxidants, enzymatic antioxidants, or combinations of these various antioxidants. Antioxidant liposomes hold great promise in the

treatment of many diseases and conditions in which oxidative stress plays a prominent role. Several studies have clearly indicated that the liposomal antioxidant formulations compared to that of the free non-encapsulated antioxidants exert a far superior protective effect against oxidative stress-induced tissue injuries.

Military Significance: The overall objective of this study is to develop an effective prophylactic therapy against CEES-induced skin damage (analogous to HD effect) based on the topical application of antioxidant liposomes. Our preliminary data suggest that antioxidant liposomes are very effective in preventing CEES toxicity to stimulated macrophages. This study will determine potential effectiveness of various liposome formulations in ameliorating the CEES-induced skin injury. The successful outcome from this research will comprise a treatment that will reduce or prevent casualties in the battlefield thus preserving combat effectiveness. Furthermore, the timely administration of this treatment regimen will also avert the devastating results of CEES against civilians that might be exposed to CEES during a terrorist attack. In this investigation, we hope to optimize the combination of antioxidants in the liposomes to achieve the maximum therapeutic effect. In addition, we will address practical issues with regards to the large scale preparation and storage of antioxidant liposomes with long-term physical, chemical and pharmacological stability.

BODY:

Originally, we proposed using EpiDerm cultured human skin tissues as a working model to study CEES cytotoxicity and protective effects of antioxidant liposomes. However, EpiDerm tissues are expensive, and in order to save resources, we have chosen cultured normal human epidermal keratinocytes (NHEK) purchased from Cambrex to use in our first series of experiments. In addition, we have used cultured immortalized human keratinocytes CCD 1106 KERTr purchased from ATCC, which are easy to culture and allow unlimited number of passages, whereas NHEK spontaneously transform after 5-7 passages (a major and costly limitation). These issues were discussed and recommended by USAMRICD scientist Dr. R. Ray who specializing in studies of HD toxicity mechanisms. We plan to collect the bulk of preliminary results with CEES (cell viability, apoptosis, cytokine release, oxidative stress parameters) and to optimize antioxidant liposome formulations using cultured human keratinocytes, and then perform final experiments with the EpiDerm tissues. As discussed below, this strategy has proven useful since we have already optimized the technique for delivering maximally toxic levels of CEES to cultured skin cells by use of dimethyl sulfoxide (DMSO).

Task 1: In these initial experiments we will characterize the toxicity of CEES to the keratinocytes or EpiDerm model as a function of CEES dose and exposure time. We will also determine: (1) how apoptosis contributes to CEES induced toxicity by measuring both capase-3 activity and DNA fragmentation; (2) if immuno-stimulators (LPS, PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) increase CEES toxicity to human keratinocytes/EpiDerm tissues.

It is postulated that apoptosis is the major cell death mechanism at low doses of CEES or HD whereas necrosis is the dominant mechanism at higher concentrations of CEES or HD and long-time exposures. We further anticipate that immuno-stimulators such as pro-inflammatory cytokines, LPS and PMA will enhance CEES toxicity to human keratinocytes.

Task 2: Similar to HD, CEES induces oxidative stress in the skin cells resulting in ROS generation, DNA damaging, protein and lipid oxidation, depletion of intracellular glutathione and vitamin E (in particular, α-tocopherol and γ-tocopherol). We will determine the influence of CEES on various oxidative stress parameters using cultured human keratinocytes and, later, the EpiDerm model. Particular emphasis will be placed on intracellular ROS monitoring, GSH/GSSG ratio, tocopherol depletion/tocopheryl quinone formation, and protein oxidation.

Task 3: We will examine the pharmacokinetics of the antioxidant liposomes following their topical application to the human keratinocytes or EpiDerm tissues. Human skin cells will be treated with antioxidant liposomes containing both water- and lipid-soluble antioxidants (bi-functional liposomes), and the antioxidant status (protein oxidation, GSH/GSSG ratio, tocopherol/tocopheryl quinone ratio) of the tissues will be measured as a function of dose and treatment time. The water-soluble antioxidants to be used are GSH, NAC and α-lipoic acid (ALA); the lipid-soluble antioxidants to be used are α-tocopherol and γ-tocopherol. We will optimize the liposome formulations in order to achieve optimal enrichment of the cells with the antioxidants.

Task 4: We anticipate that liposome-encapsulated antioxidants will be more effective than free non-encapsulated antioxidants in preventing CEES toxicity in human skin cells.
We will test the effectiveness of topical application of the optimal antioxidant liposome formulations (which will be determined in Task 3) against the CEES-induced skin damage. Cultured human keratinocytes and EpiDerm system will be utilized to reveal most protective antioxidant liposome formulations.

Task 5: We will explore the possibility of manufacturing antioxidant liposomes in large scale industrial quantities. We will further optimize antioxidant liposome formulations in order to enhance their long-term physical, chemical and therapeutic stability. The most protective antioxidant liposomes (which will be determined in Task 4) will be further characterized for the physical and chemical stability as a function of storage time and conditions. Optimal storage conditions will be found out. The liposome therapeutic effectiveness will be also monitored during long-term storage under various conditions.

Progress during fist year:

Work on this grant was substantially delayed due to issues related to the hiring of key research personnel. The PI had initiated the process of hiring a highly qualified postdoctoral researcher, Dr. Victor Paromov, in June 2005 in order to have him ready to begin work on this project at the start date. However, due to the long process of transferring of his visa status to ETSU by US

CIS, Dr. Paromov was able to start only in November 2005, effectively delaying work for more then 4 months.

Liposome stability, cost and FDA Considerations

As mentioned in the Introduction, liposomes are unique in their ability to effectively deliver both water- and lipid-soluble antioxidants to the skin cells. As liposomes are artificially made vehicles, the question of their long-term stability especially under non-optimal conditions (high temperature, dry air) is of high importance. It would not be appropriate to use unstable liposome formulations for the Task 3 and Task 4, which are of greatest importance in this project. Therefore, we addressed the question of the liposome stability at the beginning of our studies.

Relevant to **Task 5,** we have obtained the instrumentation required to prepare antioxidant liposomes in large quantities and characterize their particle size distribution. To be able to manufacture large unilamellar antioxidant liposomes in quantities up to 10-20 L per day, we have optimized a micro-fluidizing technique. Using M-110L Laboratory Microfluidizer® Processor we can produce liposomes at a rate of 270 ml/min at 18,000 PSI. This device was chosen since this technique can be scaled-up to industrial levels without changing liposomes properties/characteristics, which is important for future FDA considerations.

The PI and Dr. Hongsong Yang have been trained by the Field Representative of Microfluidics Company on the use of M-110L Laboratory Microfluidizer® Processor and the Model 380 Nicomp particle size analyzer.

A long range consideration in the formulation of antioxidant liposomes is the very high cost of highly purified phosphatidyl choline used to make antioxidant liposomes. Purified phosphatidyl choline (i.e., dipalmitoyl phosphatidyl choline) is very useful for our initial in vitro testing but would be too expensive for a commercial product useful to the DOD. The PI has, therefore, initiated conversations with the American Lecithin Co. (www.americanlecithin.com) which has extensive experience and expertise in the use of soybean phosphatidyl choline to make liposomes. This company has supplied us with 100 g samples of various soybean phospholipids for testing. Currently we have used PL 85 G phosphatidyl choline, phospholipon 90G and phospholipon 90H (fully hydrogenated) for testing in our antioxidant liposome formulations. These products possess a high quality at lower cost (than synthetic phospholipids) and relevant FDA files are on record.

In our preliminary results, we have found that alpha-tocopherol containing liposomes were effective in protecting macrophages from CEES induced cytotoxicity. α-Tocopherol is the primary form of vitamin E found in plasma and is the primary lipid soluble antioxidant in blood. We have found, however, that γ-tocopherol is taken up by cells in culture to higher extent then α-tocopherol [19]. In the experiment plan in this proposal we will test liposomes made with either α-tocopherol or γ-tocopherol or mixtures of both. γ-Tocopherol is also very expensive and a commercial antioxidant-liposome formulation based on this vitamin E isoform would be prohibitively expensive. The PI has, therefore, also made contact with commercial suppliers of mixed tocopherols with high a content of γ-tocopherol. The DSM Nutritional Products

(www.dsm.com) has supplied us with 100 g quantities of mixed tocopherols (with a very high content of γ-tocopherol) for testing in the antioxidant liposomes.

The liposome formulations have been characterized by measuring: 1) particle size distribution using a dynamic light scattering Model 380 Nicomp particle analyzer; (2) liposome antioxidant (e.g., vitamin E) content composition and stability; and (3) potential liposome cytotoxicity in human skin cells using the MTT assay (**see Task 1**). In addition, liposomes containing encapsulated water-soluble antioxidants, such as glutathione (GSH) and N-acetyl cysteine (NAC), were tested on their chemical stability (GSH oxidation) and physical stability (GSH leakiness).

The following large unilamellar liposomal (LUV) formulations were made: (1) blank liposomes (no water or lipid soluble antioxidants); (2) GSH-liposomes (75 mM GSH); (3) Toc-liposomes; (4) 2x-Toc-liposomes; (5) T/G liposomes (3.33 mM α-tocopherol with 75 mM GSH). These liposomes have been further divided into groups: (1) stored at room temperature or in refrigerator at 4°C; (2) stored with or without EDTA or urate as chelating agent. Table 1 shows the composition of the alpha-tocopherol liposomes.

Table 1-Antioxidant Liposomes

- 1 or 3 passes
- 0, 6.6 or 2x6.6 mole% alpha-toc
- Soy lecithin (90 G) from American Lecithin Company
- Stored at room temperature or 4ºC

Lipid	Mole ratio	Mole fraction
lecithin	10	0.66
cho	4	0.26
PS	0.1	0.0066
alpha-toc	1	0.066
totals	15.1	1.00

Particle size distribution: Our results with respect to size distribution are still in the process of being analyzed. To date we can tentatively conclude that: (1) typical liposome preparation mostly contains vesicles with sizes distributed within 100-300 nm interval; (2) three passes through the homogenizer produces slightly smaller but more uniform liposomes (**Figure 1**), further increase of the number of passes does not does not improve the vesicle size distribution; (3) encapsulation of a water-soluble antioxidant, such as NAC or GSH does not significantly alter the liposome size distribution; (4) addition of a lipophilic antioxidant, such as α-tocopherol, in the liposome formulation slightly changes average particle size (**Figure 2**), with high levels increasing and medium levels not having much of an effect.

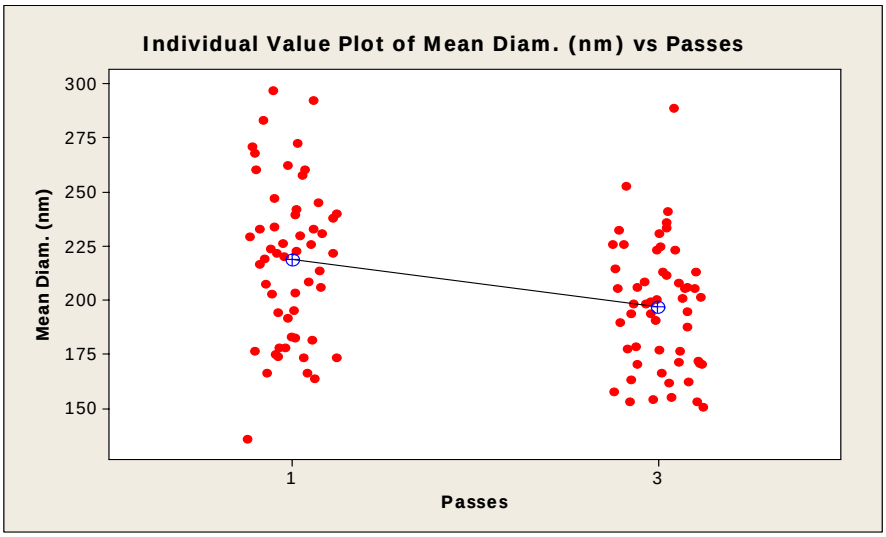

Figure 1. Three passes through the microfluidizing Processor makes smaller but more uniform liposomes. The liposomes prepared using M-110L Laboratory Microfluidizer® Processor; vesicle size distribution analyzed using the Model 380 Nicomp particle size analyzer.

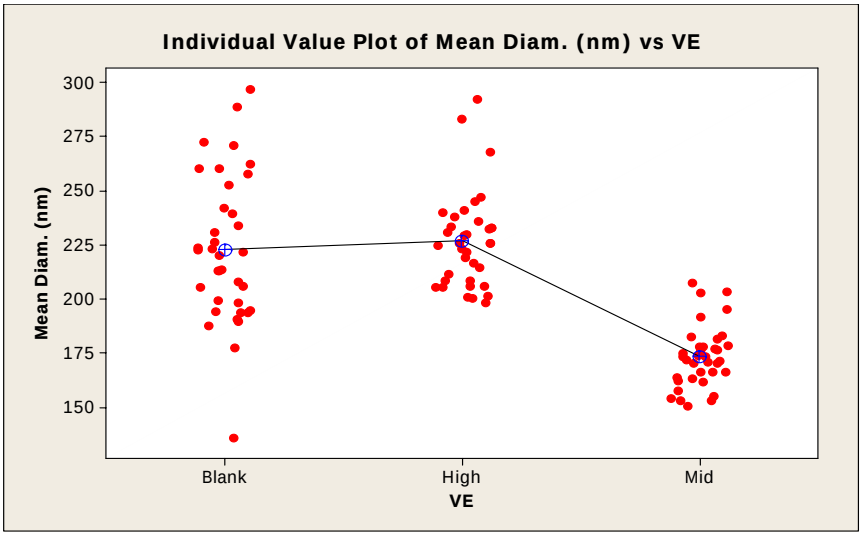

Figure 2. Vitamin E influences liposome size distribution. Blank liposomes contain no antioxidants; Mid. Vitamin E (VE) liposomes contain 6.67% mol./mol. α-tocopherol); High VE liposomes contain 13.33% mol./mol. α-tocopherol. The liposomes were prepared using a M-110L Laboratory Microfluidizer® Processor; vesicle size distribution analyzed using the Model 380 Nicomp particle size analyzer.

Antioxidant content composition and stability: We have analyzed changes in lipid composition of vitamin E containing liposomes during prolonged storage. **Table 2** shows the changes in vitamin E content of α- or γ-tocopherol containing liposomes during 4 week storage

either at room temperature (RT) or at 4° C. Tocopherols were determined within the liposome samples using HPLC with electrochemical detection. Vitamin E containing liposomes stored at RT were completely depleted of vitamin E. In addition, phase separation was also visually registered during the 4th week of storage at RT.

Table 2. Changes in the Vitamin E Content of Liposomes During Storage

Vitamin E in Liposomes (mole percent)	6.67	13.33
	Tocopherol Levels in Formulation (mM)	
Day 1	2.98 ± 0.08	4.68 ± 0.10
Day 28/RT	0.00 ± 0.01	0.00 ± 0.01
Day 28/4 deg C	0.68 ± 0.04	1.43 ± 0.15

Liposomes stored at 4° C maintained their physical stability and average particle size, however lost up to 60% of their vitamin E content due to oxidation. There were no significant differences in long-term stability between liposomes containing α-tocopherol or γ-tocopherol. We are now looking at the influence of phospholipid composition, particularly the polyunsaturated fatty acid content of the lipids to determine their influence on tocopherol stability.

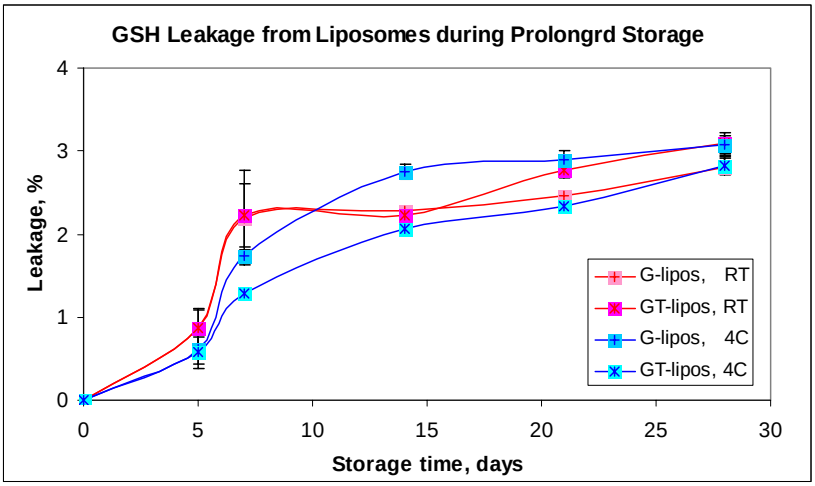

Figure 3. GSH-Liposome leakiness during pro-longed storage. 1 mL of "G-liposomes" or "G/T-liposomes" were put in sterile dialysis bag (8 kDa cut off) and dialyzed twice against 50 mL PBS. Dialyzed liposome samples were put in fresh PBS and incubated continuously at RT or at 4 C (fridge). GSH concentration within the each liposome sample and in the buffer was measured during 4 week incubation. Liposome leakiness was expressed as a percent of GSH in the buffer from the total GSH of each liposome sample.

Also we analyzed the leakiness of glutathione (GSH), a water-soluble encapsulated antioxidant, during prolonged storage (see **Figure 3).** In this experiment, GSH was encapsulated within the liposomes, and the external, non-encapsulated GSH removed by exhaustive dialysis. The liposomes were then stored either at RT or at 4° C, and GSH concentration in the buffer monitored using Tietze's method [20]. GSH leakage from the liposomes to the buffer is a measure of liposomal membrane stability as GSH molecules (charged) cannot penetrate through an intact lipid bilayer.

Our results show that the liposomes maintained high physical stability within at least 1 month of storage. Only 3% of the GSH content was lost from the GSH or G/T-containing liposomes. The loss of small amount of GSH from liposomes might be explained by the fact that the vesicles are not uniform. Although most of the vesicles have sizes within 100-200 nm intervals, actual size of some particles may differ significantly. Vesicles with variable sizes can fuse and divide spontaneously loosing their encapsulated content. However, as it is clear from **Figure 3** such perturbations are relatively minor.

In addition we analyzed chemical stability of the water-soluble content (GSH) encapsulated within the liposomes. **Figure 4** shows the loss of GSH due to oxidation to form GSSG (in GSH equivalents) as a function of storage time for the GSH-liposomes and the GSH/α-Tocopherol (G/T-) containing liposomes at RT or 4° C. These data show that GSH is oxidized fairly rapidly at room temperature compared to 4° C.

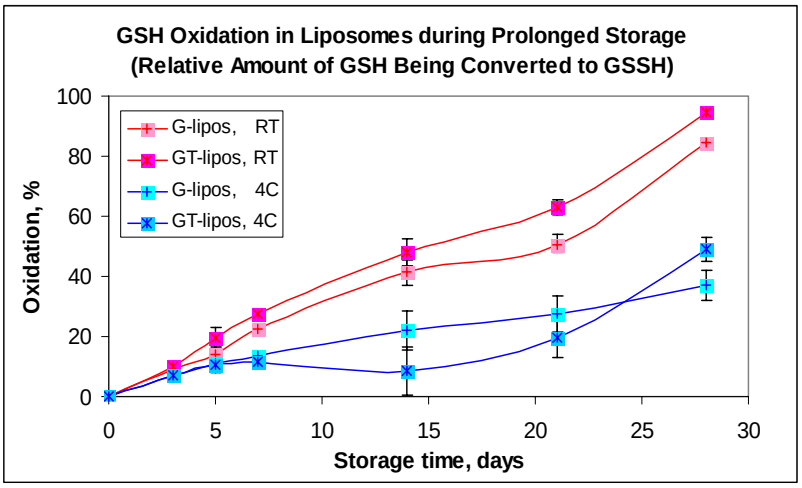

Figure 4. GSH oxidation (conversion to GSSG) within liposome fraction during pro-longed storage. "G-liposomes" or "G/T liposomes" were stored at room temperature (RT) or at 4 C. GSH and GSSG concentrations within the liposome samples was measured after 3, 5, 7, 14, 21 and 28 days of storage. Oxidation of GSH content of the liposomes was expressed as a percent of GSH converted to GSSG relatively to the total GSH/GSSG content of each sample.

Moreover, the presence of α-tocopherol does not retard GSH oxidation. In addition, G/T-liposomes, similar to α-tocopherol containing liposomes, loose their physical stability (visible phase separation) during the 4th week of storage at RT.

The experiments describe above were done in the absence of a metal ion chelator. Chelators act as antioxidants by minimizing oxidation catalyzed by iron ions (a contaminant in all buffers). We have, therefore, analyzed the influence of metal ion chelators (EDTA and urate) on GSH chemical stability within the liposomes. **Figure 5** and **Figure 6** show the effect of the chelators on GSH oxidation within the G- or G/T- liposomes. Neither urate nor EDTA protected the GSH content of the liposomes during storage at 4° C. However, both chelators slightly protected the GSH of G/T-liposomes but not that of the G-liposomes stored at RT (**Figure 7**).

Based upon the above studies we concludes that antioxidant liposomes posses a high degree of physical stability even at RT. We found that the loss of anti-oxidative potency of the liposomes due to the chemical oxidation of the antioxidants is a major difficulty during long-term storage. We anticipate that NAC and alpha lipoic acid (ALA) would be more stable than GSH. Clearly, the storage temperature is emerging as a key factor in long term stability. Both lipid- and water-soluble antioxidant contents are being preserved much better at 4° C. These data suggest that GSH and/or vitamin E containing liposomes would have to be either used relatively soon after being formulated (perhaps within a week) if stored at RT or could be stored at 4° C for up to 4 weeks.

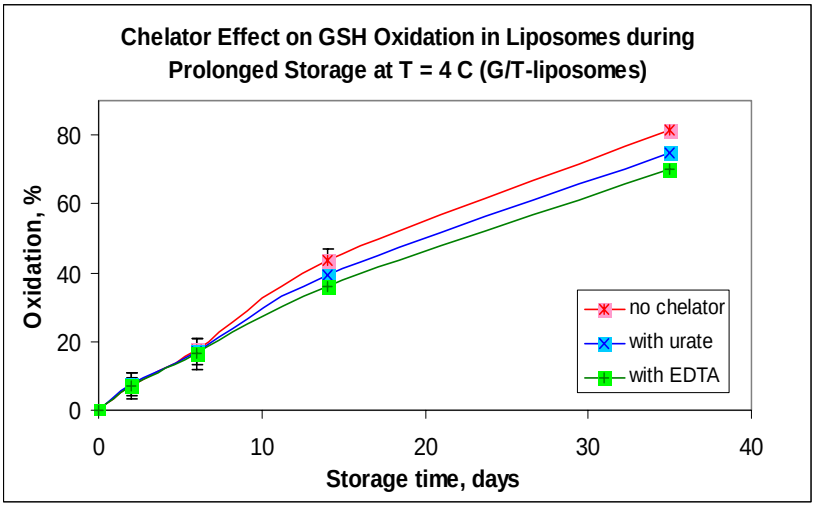

Figure 5. Chelator effect on GSH oxidation (conversion to GSSG) within liposome fraction during pro-longed storage. "G/T-liposomes" were stored at 4C (fridge). GSH and GSSG concentrations in the liposome samples was measured after 2, 6, 14, and 35 days of storage. Oxidation of GSH content of the liposomes was expressed as a percent of GSH converted to GSSG relatively to the total GSH/GSSG content of each liposome sample.

Cytotoxic Effect of CEES On Cultured Human Keratinocytes

Task 1: We have investigated cytotoxic effect of CEES on cultured human keratinocytes.

Figure 7 shows our initial attempts at defining the dose and time response of the NHEK keratinocytes to CEES. In these experiments, the CEES was first dissolved in ethanol and then added to the culture medium with the final concentration of ethanol being no more than 2% by volume.

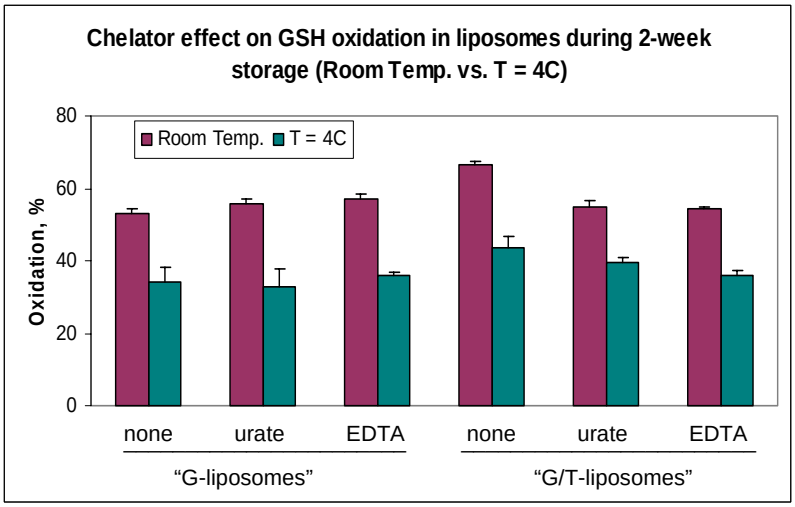

Figure 6. Chelator effect on GSH oxidation (conversion to GSSG) within liposome fraction during 2-week storage. "G-liposomes" or "G/T-liposomes" were stored at RT or 4 C (fridge). GSH and GSSG concentrations in the liposome samples was measured after 14 days of storage. Oxidation of GSH content of the liposomes was expressed as a percent of GSH converted to GSSG relatively to the total GSH/GSSG content of each liposome sample.

Figure 7 shows cell viability (as measured by the MTT assay) as a function of CEES dose after 16 or 48 hours of incubation. We found that NHEK cells were much more resistant to CEES induced inhibition of cell growth than other cell lines tested with a similar experimental design. As anticipated, higher doses and longer time of CEES incubation resulted in increased growth inhibition.

In order to determine if an addition of immuno-stimulators such as lipopolysaccharide (LPS) or phorbol myristate acetate (PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) increase CEES toxicity to human keratinocytes, we performed further experiments with NHEK cells exposed to various levels of CEES (**data not shown**). None of these immuno-stimulators enhanced toxicity of CEES as measured by the MTT assay. These results sufficiently differ with our previous results obtained with murine macrophages simultaneously exposed to CEES and LPS [5].

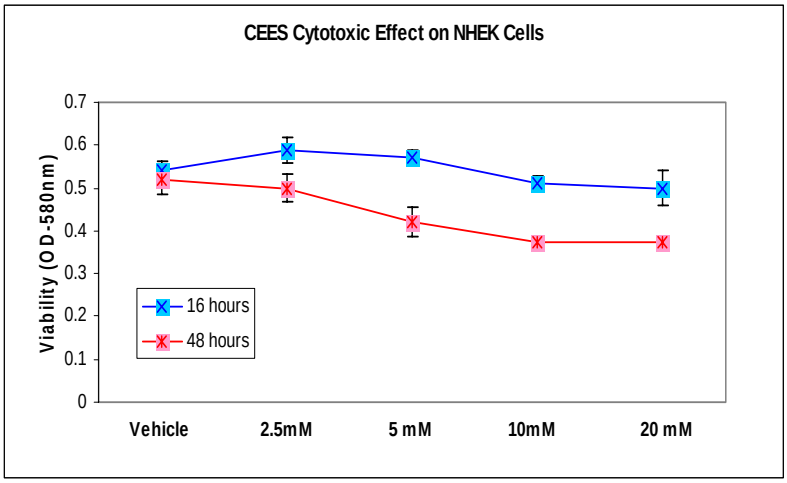

Figure 7. Cytotoxic effect of CEES on NHEK cells during long-time incubations. NHEK cells (Cambrex) were treated with various levels of CEES for up to 48 hours. CEES was applied as a stock solution in ethanol (final concentration of ethanol was no more than 2% vol.). Cell viability was measured as absorbance at 580nm (standard MTT assay).

The fact that LPS and other stimulators do not alter CEES toxicity in human keratinocytes possibly reflects a multi-step reaction to CEES/HD toxicity in human, which involves at least two major types of cells – skin cells and immune cells. We anticipate that, in the simplest case, CEES promotes cytokine release in keratinocytes which then induces the activation of macrophages and other immune cells (mast cells for instance),

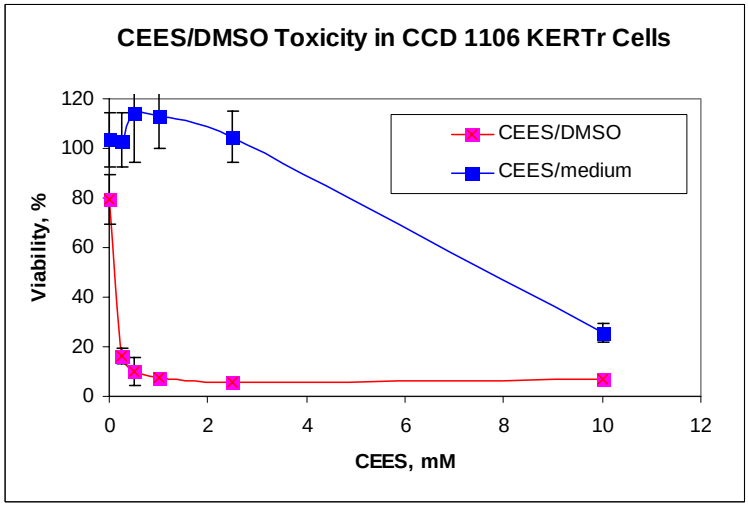

Figure 8. Cytotoxic effect of CEES/DMSO on NHEK cells during 24 h incubation. CCD 1106 KERTr cells were placed into 96-well plate in 0.25 ml/well of medium and treated with CEES applied either as pre-mixed solution in the media or as a stock solution in DMSO (final conc. 2% vol/vol). Cell viability was measured as absorbance at 580nm (standard MTT assay).

which in turn produce a feed-back reaction in keratinocytes stimulating apoptosis [10, 11, 14, 21].

In the experiments described above we have applied CEES in ethanol. According to the USAMRICD studies, human skin exposed to 300 µM concentration of HD will demonstrate oxidative stress, cytokine release, and massive apoptosis followed by necrosis. As a less toxic analog of HD, CEES would be expected to produce similar changes in keratinocytes at concentrations 3-4 times higher, i.e., 1 mM [22].

We, however, did not observe substantial cell death in human keratinocytes exposed to fairly high levels of CEES applied as a stock solution in ethanol. This method of CEES application appeared to result in a phase separation of the CEES from the cell culture medium thereby decreasing its effective concentration. We have discussed this difficulty with USAMRICD specialist Dr. R. Ray at the Bioscience Review 2006 Conference. Dr. R. Ray has recommended two alternative methods of CEES application. The first method requires directly adding CEES to a plastic tube with the culture medium followed by extensive vortexing and immediately applying this mixture to cells in a multi-well plate. The second method requires preparation of a CEES stock solution in dimethyl sulfoxide (DMSO) which is then added to cells in a multi-well plate with gentle mixing. DMSO is known to be an very effective vehicle for a delivery of organic compounds to skin and enhances HD toxicity.

We have tried both these alternative methods of CEES application and the results as shown in **Figure 8 (see above)**. **Figure 9** shows the dose-response at lower levels of CEES. CEES directly mixed with medium and applied to the keratinocytes showed moderate toxicity. In marked contrast, CEES in DMSO was much more toxic. We will, therefore, optimize CEES/DMSO method of application in our next series of experiments looking at the potential protective effect of antioxidant liposomes (Tasks 3 and 4). Pre-treatment of cells with DMSO alone for 2 hours did not potentiate the toxicity of CEES (in medium) suggesting that DMSO is only acting to improve the solubility of CEEES.

In addition to the above described studies we will monitor cytokine release (particularly IL-8 and TNF-α) as they have been found to be superior markers for CEES induced inflammation. Similarly, markers of apoptosis (caspase 3 and DNA fragmentation) will be measured in future experiments.

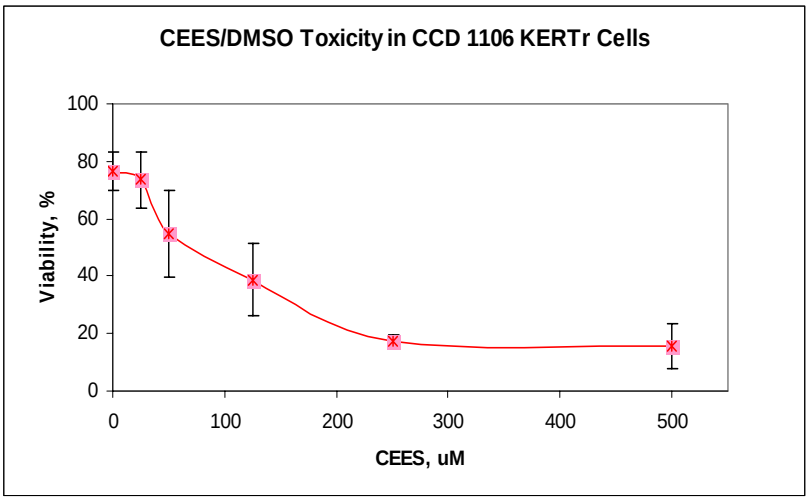

Figure 9. Cytotoxic effect of CEES/DMSO on NHEK cells during 24 h incubation. CCD 1106 KERTr cells were placed into 96-well plate in 0.25 ml/well of medium and treated with CEES applied as a stock solution in DMSO (final conc. 2% vol/vol). Cell viability was measured as absorbance at 580nm (standard MTT assay).

Oxidative Stress Parameters in CEES Treated Human Keratinocytes

Task 2: We have done a few preliminary experiments to monitor oxidative stress parameters in CEES (ethanol) treated human keratinocytes. **Figure 10** shows the generation of intracellular superoxide anion (O_2*) in NHEK cells during 4 hours of incubation with 10 mM CEES. Superoxide levels were measured using a specific fluorescent dye, dihydroethidium (HEt). Intracellular oxidation of HEt (measured as fluorescence of the product) reflects superoxide generation in cytoplasm. NHEK cells were loaded with 10 µM HEt for 1 hour, washed, and exposed to CEES. As was expected, 10 mM CEES induced high levels of superoxide production in NHEK cells.

Interestingly, the production superoxide anion in NHEK cells appears to increase in a similar manner as CEES induced toxicity. These data confirm that oxidative stress, and ROS generation in particular, is an important factor in CEES induced cell death.

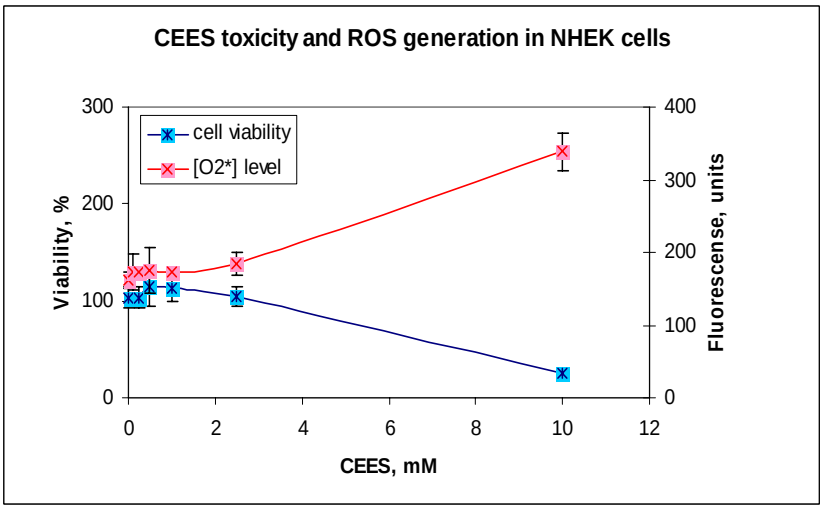

Figure 10. O_2* generation (measured as HEt intracellular oxidation) in NHEK cells after 4 h incubation was compared to CEES toxicity after 24 h. NHEK cells were loaded with 10 uM HEt for 1 h, washed by Hank's salt solution, and treated with CEES/ethanol. HEt oxidation was measured (end-point assay) as fluorescence at 360nm/410nm (ex./em.). Cell viability was measured as absorbance at 580nm (standard MTT assay).

We also measured intracellular production of hydrogen peroxide (H_2O_2) in CEES/ethanol treated NHEK cells (**Figure 11**). The cells were loaded with 20 µM dichlorofluorescein diacetate (DCFH-DA) for 1 h, then washed, and exposed to CEES. In the cytoplasm, DCFH-DA is rapidly converted to dichlorofluorescein (DCFH) by esterases. H_2O_2 selectively oxidizes DCFH yielding a highly fluorescent product. Surprisingly, CEES diminished hydrogen

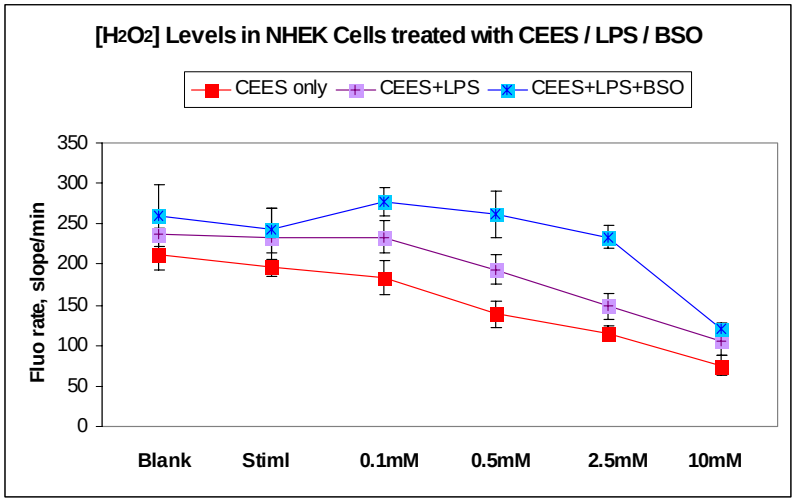

Figure 11. H_2O_2 generation (measured as DCFH-DA intracellular oxidation) in NHEK cells during 4 h incubation with CEES/ethanol. NHEK cells were loaded with 20 uM DCFH-DA for 1 h, washed by Hank's salt solution, and treated with CEES; or CEES and100 ug/mL LPS; or CEES and 100 ug/mL LPS, and 100 uM BSO (as indicated). DCFH-DA oxidation was continuously monitored (kinetic assay) as fluorescence at 485nm/520nm (ex./em.).

peroxide production in human keratinocytes. However, incubation of the cells with 50 μM H_2O_2 as a positive control did show increased levels of hydrogen peroxide (**data not shown**). We also determined if stimulation of the cells with LPS or pretreated with buthionine sulfoximine (BSO) altered H_2O_2 production (**Figure 11**). HNEK cells stimulated with LPS or pretreated with buthionine sulfoximine (BSO) showed increased levels of H_2O_2. Pretreatment of the cells with BSO blocks GSH synthesis.

The decreased oxidation of DCFH at higher CEES levels can be potentially explained by the fact that this dye is also sensitive to intracellular NO. Previously, we have found that CEES inhibits NO production in murine macrophages [6]. It is possible that a similar effect occurred in the human keratinocytes. Additional experiments to measure NO production in keratinocytes will be needed to explore this possibility.

Task 3: Measurements of α-tocopherol and GSH uptake by human keratinocytes after incubation with antioxidant liposomes are currently in progress.

Task 4:

We have initiated experiments with four different types of antioxidant liposomes in order to test their protective abilities against CEES toxicity. **Figure 12** shows an effect of NAC/T-liposomes (**see description on page 9**) while NHEK cells were incubated for 24 hours with 10 mM CEES applied as a stock solution in ethanol. Interestingly, NAC/T-liposomes were not only protective, but also stimulated proliferation of NHEK cells both in the presence and in the absence of CEES. Blank liposomes (vesicles of similar size distribution composed only with phospholipids and cholesterol, but not containing antioxidants) did not show any protective effect, as was expected.

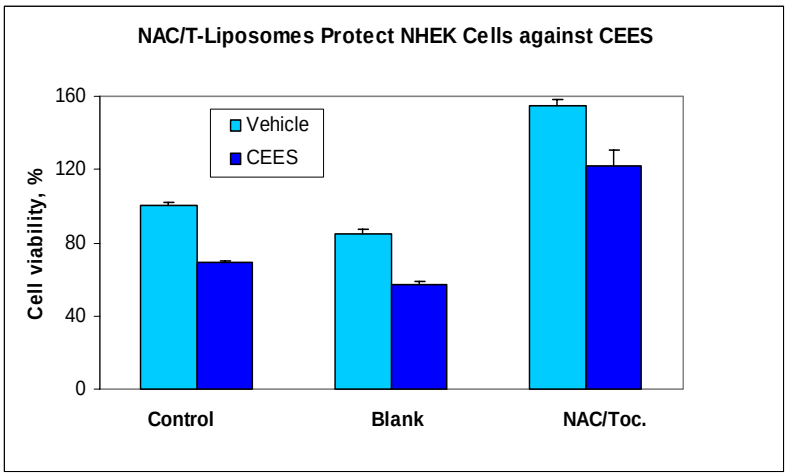

Figure 12. Protecting effect of NAC/T-liposomes (NAC/Toc) was compared to the effect of Blank liposomes (Blank) on NHEK cells treated with 10 mM CEES for 24 h. CEES was applied as a stock solution in ethanol. Control: cells treated with ethanol or CEES in the absence of liposomes. Cell viability was measured as absorbance at 580nm (standard MTT assay).

Figure 13 illustrates the influence of Blank liposomes, G-liposomes, G/T-liposomes, or T-liposomes on NHEK cell growth inhibition after either 24 or 48 hour of incubation with 10 mM CEES/ethanol. The highest cytotoxic effect of CEES (71% of control viability) was detected

after 48 hours of incubation with CEES. After 48 hours blank-liposomes were not protective against

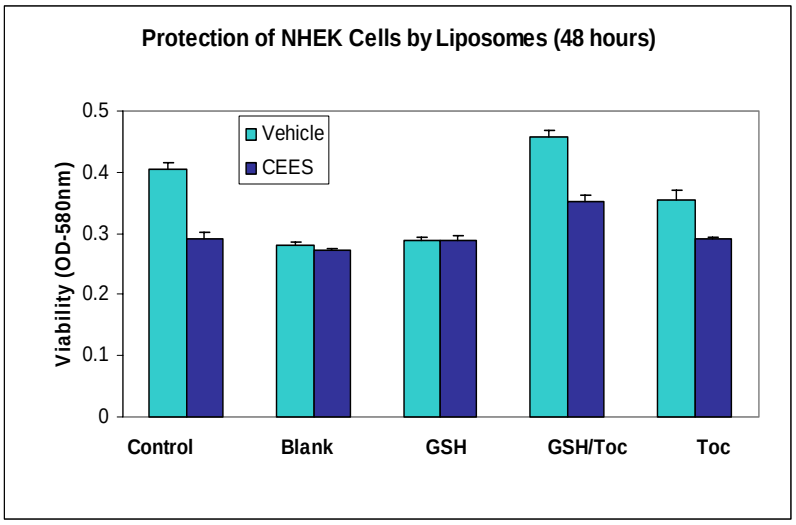

Figure 13. Protecting effect of antioxidant liposomes in NHEK cells treated with 10 mM CEES/ethanol for 48 hours. Control: cells treated with ethanol or CEES in the absence of liposomes; Blank: Blank liposomes; GSH: G-liposomes; GSH/Toc: G/T-liposomes; Toc: T-liposomes. Cell viability was measured as absorbance at 580 nm (standard MTT assay).

CEES (67%) and decreased cell viability to 69% without CEES; GSH containing liposomes were also not protective against CEES (71%) and decreased cell viability to 71% without CEES; alpha-tocopherol containing liposomes were also not protective against CEES (72%) and decreased cell viability to 88% without CEES; only liposomes containing both GSH and alpha-tocopherol were protective against CEES (87%) and, surprisingly, increased cell proliferation without CEES to 113%.

Interestingly, antioxidant liposomes not only show protective effect against CEES-induced cell damage in keratinocytes, but also stimulate the cell growth and proliferation. These experiments are need to be repeated using DMSO as a vehicle for CEES in order to achieve its maximum cytotoxic effect in human keratinocytes, allowing us to test antioxidant liposomes under the most stringent conditions. We will also be measure cytokine release (TNF-α, IL-8, IL-6) and apoptosis indices (caspase 3, DNA fragmentation) monitoring as additional biomarkers of CEES/DMSO induced cell damage.

KEY RESEARCH ACCOMPLISHMENTS:

- □ Physical and chemical stability of antioxidant liposomes during long-term storage have been tested
- □ Various aspects of CEES toxicity in human keratinocytes (inhibition of cell growth, oxidative stress parameters, influence of various immuno-stimulators) have been studied
- □ Major types of antioxidant liposomes have been tested in prevention of CEES-induced cell damage in human keratinocytes; protective/stimulating effects have been documented
- □ Results have been presented as a poster/abstract at the Bioscience Review 2006 International Conference Hunt Valley, Maryland

REPORTABLE OUTCOMES:

Publications:

Suntres, Z, Stone, WL, Smith, MG: Ricin-Induced Toxicity: The Role of Oxidative Stress, J Med CBR Defense, 3 (2005).

Smith, M, Das, S, Ward, P, Suntres, Z, Crawford, K and Stone, WL: Blister Agents and Oxidative Stress, In: CHEMICAL WARFARE AGENTS: Chemistry, Pharmacology, Toxicology and Therapeutics, CRC Press (in preparation)

Presentations/Abstracts:

Hoesel, L.M., Pianko, M.J., Yang, H., Stone, W.L., Smith, M.G., Ward, P.A., Liposomes Containing Antioxidants Prevent Pulmonary Fibrosis in Half-Sulfur Mustard Gas Induced Lung Injury, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, invited platform presentation and abstract (page 109).

Stone, W.L., Li, Q., Paromov, V., Qui, M., Yang, H., Smith, M., Antioxidant Liposome Therapy for Exposure to a Sulfur Vesicating Agent, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, poster presentation and abstract (page 161).

Smith, M.G., Stone, W.L., Ward, P., Alibek, K., Wu, A., Das, S., Crawford, K., Anderson, D., Sciuto, A., Suntres, Z., Rest, R., A Multi-Threat and Diagnostic Countermeasure by the Advanced Medical Countermeasure Consortium, Bioscience Review 2006, sponsored by the US Army Medical Research and Material Command and hosted by the US Army Medical Research Institute of Chemical Defense, June 4-9, 2006, Hunt Valley, poster presentation and abstract (page 259).

Degrees obtained that are supported by this award: A graduate student (Mr. Christian Muenyi, MS, Chemistry Department at East Tennessee State University) has initiated proteomic studies to complement the work funded by this application.

Funding applied for based on work supported by this award: NA

Employment or research opportunities applied for and/or received based on experience/training supported by this award: NA

Invited Presentations at International Meetings: The experiments described in this report have been presented as a poster at the Bioscience Review 2006 International Conference, Hunt Valley, Maryland (see attached abstract). In addition, Dr. Hongsong Yang has been invited to present our finding at the 6th International Workshop of Micronutrients, Oxidative Stress and the Environment held in Kuching, Malaysia, June 29th –July 2nd, 2006.

CONCLUSION:

Based upon the results described above, we conclude that antioxidant liposomes posses a high degree of physical stability even at RT. We found that the loss of anti-oxidative potency of the liposomes due to the chemical oxidation of the antioxidants is the major difficulty during long-term storage. We anticipate that NAC and ALA would be more stable than GSH. Clearly, storage temperature has emerged as a key factor in long term stability. Both lipid- and water-soluble antioxidant contents are preserved much better at 4° C. These data suggest that GSH and/or vitamin E containing liposomes would have to be either used relatively soon after being formulated (perhaps within a week) or could be stored at 4° C for up to 4 weeks.

Experiments further demonstrating CEES induced oxidative stress in human keratinocytes have been initiated. CEES induces generation of superoxide radicals and the increase in CEES toxicity has a pattern that parallels the intracellular accumulation of superoxide. Influence of immuno-stimulators (LPS, PMA) and pro-inflammatory cytokines (TNF-α, IL-1β) on CEES toxicity (in ethanol) to human keratinocytes has been tested. In these initial experiments, none of the immuno-stimulators enhanced CEES toxicity as measured by the MTT assay. Measurement of

other oxidative stress parameters (protein oxidation, GSH depletion), and monitoring of apoptotic markers (caspase 3, DNA fragmentation) and pro-inflammatory cytokines (TNF-α, IL-8, IL-6) are currently in progress.

NAC/α-tocopherol containing liposomes showed significant protective effect against CEES (in ethanol) induced skin cell damage in our preliminary experiments. GSH/α-tocopherol containing liposomes also showed some protective effect. However, these experiments needed to be repeated using DMSO as a vehicle for CEES in order to achieve its maximum damaging effect in human keratinocytes allow us to test antioxidant liposomes under the most toxic conditions.

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Appendices: Abstract for Bioscience 2006 Meeting

Antioxidant Liposome Therapy for the Exposure to a Sulfur Vesicating Agent

William L. Stone[1], Qian Li, Victor Paromov, Min Qui[1], Hongsong Yang[1], and Milton Smith[2.]

[1]Department of Pediatrics, East Tennessee State University, Johnson City, Tennessee, 37614-0578, USA, [2]Amox LTD, 27454 Rolling Pines, Lawton, MI 49065, Vienna, USA

Abstract

Vesicants are known to induce inflammation and inflammatory processes that are associated with oxidative stress and the production of reactive oxygen (ROS) and reactive nitrogen oxide species (RNOS). Oxidative stress is a physiological condition in which the production of damaging free radicals exceeds the in vivo capacity of antioxidant protection mechanisms to prevent pathophysiology. ROS and RNOS can cause pathophysiology by directly damaging biomolecules such as lipids and proteins, thereby altering their function(s). We have found that oxidative stress and inflammatory agents play a key role in the toxicity of 2-chloroethyl ethyl sulfide (CEES). CEES is a monofunctional analog of sulfur mustard (bis-2-(chloroethyl) sulfide or HD) which a bifunctional vesicant and a chemical warfare agent. Both HD and CEES are known to provoke acute inflammatory responses in skin. In particular, we have found that murine macrophages exposed to CEES have a decreased level of intracellular GSH, which is even further diminished in the presence of inflammatory agents. Pretreatment of the macrophages with N-acetyl cysteine (NAC) protects against the loss of intracellular GSH. NAC, the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl (SH) groups. NAC is converted in the body into metabolites capable of stimulating GSH synthesis, promoting detoxification, and also acts directly as a free radical scavenger. NAC has also been used to protect against the lethality of HD. We have also found that stimulated RAW264.7 macrophages exposed to 500 microM CEES (24 hours) show increased levels of protein carbonyls. Protein carbonyls are perhaps the best index for assessing oxidative stress.

These data suggest that antioxidant liposomes containing combinations of water and lipid soluble antioxidants may provide a unique therapeutic strategy for mustard gas by inhibiting the pathophysiology due to vesicant induce inflammation and oxidative stress. In this report we describe that characterization of large unilamellar antioxidant liposomes prepared using a M-110L Laboratory Microfluidizer® Processor (at a rate of 270 ml/min at 18,000 PSI). The liposomes have been characterized by measuring: 1) particle size distribution using a dynamic light scattering; (2) liposome antioxidant (e.g., vitamin E content) and stability; (3) potential cytotoxicity using the MTT assay and; (4) cellular antioxidant uptake in RAW264.7 murine macrophage cell line; (5) ability to prevent CEES toxicity to NHEK adult keratinocytes.

Our results with vitamin E-liposomes to date indicate that: (1) the vitamin E content of liposomes can alter their size distribution; (2) neither the temperature of storage (4 ^{o}C or room temperature) nor the storage time (up to one month) influences the mean liposome diameter; (3) the vitamin E content of the liposomes decreases to very low levels when stored at room temperature for one month but this effect is much less pronounced at 4 ^{o}C (4) the antioxidant liposomes are not cytotoxic to RAW264.7 cells; (4) the vitamin E content of RAW264.7 macrophages dramatically increases by incubation (for 24 hours) with vitamin E containing liposomes present in the medium at levels up from 15 to 55 microM (the range tested). Physiological levels of vitamin E are in the 10-30 microM range.

This work was supported by the U.S. Army Medical Research and Materiel Command under Contract/Grant/Intergovernmental Project Order DAMD17-98164001 and Contract W81XWH-05-2-0034.